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Temperature and viscosity dependence of the electron-transfer reaction between plastocyanin and cytochrome *c* labeled with a ruthenium(II) bipyridine complex

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Abstract

The temperature and viscosity dependence of the photo-induced electron-transfer reaction between plastocyanin and cytochrome *c* labeled at Lys13 with Ru(4,4′-dicarboxybipyridine)(bipyridine)$_2^{+2}$ have been investigated. In these studies, a short pulse of 450 nm light was used to excite the ruthenium complex which was oxidatively quenched by the iron center of cytochrome *c*. The resulting Fe(II) cytochrome *c* was then rapidly reoxidized by plastocyanin. The reactions were investigated over a temperature range of 3.5 to 37°C under low ionic strength conditions such that protein/protein complex formation was favored. The enthalpy of activation was 7 kcal mol^{-1} and the entropy of activation was −20 cal mol^{-1} K^{-1}. Increasing the viscosity by the addition of sucrose up to 70% resulted in a 4-fold decrease in the rate constant for electron transfer. The overall results suggest a rate-limiting step that involves either dissociation of the dominant protein/protein complex or surface diffusion of the associated proteins.

Keywords: Plastocyanin; Cytochrome *c*; Electron transfer; Kinetics

1. Introduction

The reaction between cytochrome *c* (CC) and plastocyanin (PC) has provided a useful and popular model system for the study of interprotein electron transfer [1,2]. Although the two proteins are not physiological reaction partners, their use in such studies is, in part, due to the fact that both are structurally well characterized [3–5]. The association of plastocyanin with cytochrome *c* has been extensively examined by NMR [6,7] and has been the focus of a detailed computer modeling study [8]. A number of investigators have explored the kinetics of electron transfer [9–12] between cytochrome *c* and plastocyanin. Peerey and Kostić, for example, used pulse radiolysis to measure the rate constant for electron transfer in the CC/PC complex. These investigators also studied the photoinduced electron-transfer reactions of zinc [13] and tin [14] substituted cytochromes *c* with plastocyanin. Additional studies focused on the electron-transfer reactions of covalently cross-linked CC/PC complexes [13].

Studies to date [8] suggest that plastocyanin forms an electrostatically stabilized protein/protein complex with cytochrome *c*. The binding domain on plastocyanin appears to involve several acidic residues, 42–45 and 59–61, which cluster around

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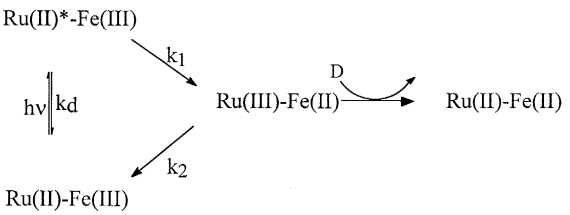
Tyr83. The copper site is near the surface at the top of the protein which is predominantly hydrophobic and approx. 12 Å away from Tyr83. The question of whether the most favorable binding site is actually the site at which electron transfer takes place is controversial and is the focus of the experiments to be described. Zhou and Kostić [14] have shown with zinc substituted cytochrome *c*, that electron transfer is rate-limited by a process which is viscosity-dependent. Kostić suggested that this process involves 2-dimensional diffusion over the surface of the protein within a solvated protein/protein complex. These investigators [11,12] have also shown that electron transfer does not occur in a covalently linked protein/protein complex with iron containing cytochrome *c* but does in the zinc-substituted analogue.

Covalent attachment of photoactive ruthenium complexes to CC has provided a valuable tool for investigating the electron-transfer reactions of cytochrome *c* and its reaction partners [15]. Flash photolysis of the Ru(II)-CC(III) form of the Ru-CC derivatives produces an excited state Ru(II)*-CC(III) which rapidly transfers an electron to the Fe(III) center of CC to yield Ru(III)-CC(II). Electron transfer from the CC(II) to other redox partners can be observed if an appropriate sacrificial electron donor is present in solution. The sacrificial donor reduces Ru(III) back to Ru(II). The reaction sequence is shown in Scheme 1.

We initially demonstrated the feasibility of this approach using the CC/PC system [10]. In that study, we demonstrated that (1) electron transfer from CC to PC could be photochemically initiated by flash photolysis of ruthenium(II) polypyridine complexes covalently attached to CC, (2) that the dependence of the observed rate constant on [PC] could be used to determine both K_a, the association constant for the complex between the two proteins, and k_{et}, the first-order rate constant for electron transfer within the protein-protein complex, (3) that at high ionic strengths the decrease in k_{obs} was consistent with the dissociation of electrostatically stabilized complex between PC and CC.



Scheme 1.

In order to further explore the features which control the electron-transfer reaction between iron containing CC and PC, we have examined the temperature and viscosity dependence of the electron-transfer reaction between PC and cytochrome *c* labeled at Lys13 with a derivative of $Ru(bpy)_3^{+2}$.

2. Materials and methods

2.1. Materials

Horse heart cytochrome *c* labeled at Lys13 with $Ru(bipyridine)_2(dicarboxybipyridine)^{2+}$ was available from previous studies. The preparation has been described by Pan et al. [15]. Plastocyanin was isolated from spinach as described by Geren et al. [16].

2.2. Kinetic measurements

The flash photolysis system, kinetic measurements and analysis have been described by Pan et al. [15]. In the present experiments the sample solutions (300 μl) were placed in glass semi-microcuvettes which were held in a water-jacketed cell holder. Water from a Neslab RTE-8 refrigerated temperature bath was circulated through the cell holder to maintain constant temperature. Temperature was measured at the cell holder with a type J thermocouple and an Omega Model 680 digital thermometer. Experiments showed that the temperature of the cell holder and the sample were identical 20 min after the bath reached any temperature within the range of the reported experiments. Sample solutions contained 5 μM CC, 5–40 μM PC, 20 nM cytochrome oxidase, 0.5 mM EDTA or 10 mM aniline and were buffered with phosphate buffer. Direct measurements of the sample solutions showed that the pH of all of the solutions was 7.0 ± 0.1.

The concentrations of reduced and oxidized cytochrome *c* were monitored at 550 nm and that of oxidized plastocyanin at 600 nm. The extent of the changes in the redox states of the two proteins were

calculated using the following differences in extinction coefficients; 18.7 M^{-1} for CC(II)/CC(III) at 550 nm [16] and 4.9 M^{-1} for PC(I)/PC(II) at 600 nm [17]. When aniline was used as a sacrificial agent, measurements were complicated by absorbance due to the aniline cation radical intermediate or products of its decomposition. In these experiments only data taken at 550 nm were used. All kinetic data were fit to single exponential equations using a weighted least-squares procedure. The correlation coefficients for the fitted data were greater than 0.99 with a standard deviation of the mean of the observed rate constants obtained from independent data sets of 10% or less.

The temperature dependence was fit to the Eyring equation

$$\ln(k_{et}/T) = \Delta S^{\pm}/R + \Delta H^{\pm}/RT \quad (1)$$

where the constants have their usual values. The standard deviation of the activation parameters based on repeated experiments is conservatively estimated at 15%.

$\Delta H^{0\prime}$ for the electron-transfer reaction between CC and PC was calculated from the relation $\Delta G^{0\prime} = \Delta H^{0\prime} - T\Delta S^{0\prime}$. $\Delta G^{0\prime}$ was calculated from the $E_{1/2}$'s. $\Delta S^{0\prime}$ was obtained from the temperature coefficients of the $E_{1/2}$'s reported by Nocera et al. [18] for CC and Armstrong et al. [19] for PC. From this data $\Delta G^{0\prime} = -2.9$ kcal mol^{-1}, $\Delta H^{0\prime} = -0.8$ kcal mol^{-1} and $\Delta S^{0\prime} = 7$ cal mol^{-1} K^{-1}.

The viscosity dependence of the rate constants was determined by adding sucrose to the reaction mixtures. The sucrose concentration was varied from 0 to 70%. The viscosity of the solutions was determined from the measured density using standard tables [20].

3. Results

Flash photolysis of Ru-13-CC(III) in the presence of PC and a sacrificial donor such as aniline or EDTA leads to the rapid formation of Ru-13-CC(II). Subsequently, Ru-13-CC(II) is slowly oxidized by electron transfer to PC as indicated in Scheme 1. In our initial study [10] we reported only transient absorbance measurements recorded at 550 nm which are indicative of the redox states of cytochrome *c*. We were not able to monitor redox changes at the copper center because of instrumental limitations. Recent measurements [21] have also shown that the aniline cation radical or products of its decomposition have a broad absorbance which complicates analysis of the weak transient absorbance expected for PC at 600 nm. We have now demonstrated that the electron is indeed going from CC to PC by following the reduction of PC at 600 nm in a system using EDTA as the sacrificial electron donor. Absorbance transients recorded at 550 nm and 600 nm are shown in Fig. 1. The rate constants determined from data collected at 550 nm and 600 nm are identical within experimental error. In addition, the magnitude of the changes in absorbance at 550 nm and 600 nm indicate that oxidation of cytochrome *c* is accompanied by a molar equivalent reduction in PC. Identical rate constants (within 95% confidence limits) were obtained with either 10 mM aniline or 0.5 mM EDTA as sacrificial electron donors when experiments were performed at equivalent ionic strengths.

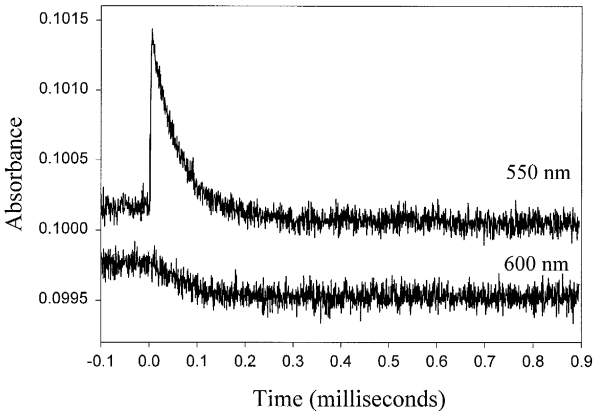


Fig. 1. Transient absorption spectra of the photoinduced reaction of 5 μM Ru-13-cytochrome *c* and 40 μM plastocyanin with 500 μM EDTA and 5 mM phosphate buffer (pH = 7) monitored at 550 and 600 nm.

Fig. 2 shows the dependence of the observed rate constants on the concentration of PC under different solution conditions. The first-order rate constant, k_{et} and the association constant, K_a were determined by fitting k_{obs} to Eq. (2) as previously described [10].

$$k_{obs} = k_{et}K_a[PC]/(1 + K_a[PC]) \quad (2)$$

The curve fitting was done using the Marquart-Levenberg method contained in the program PSI-PLOT from Software International. The resulting val-

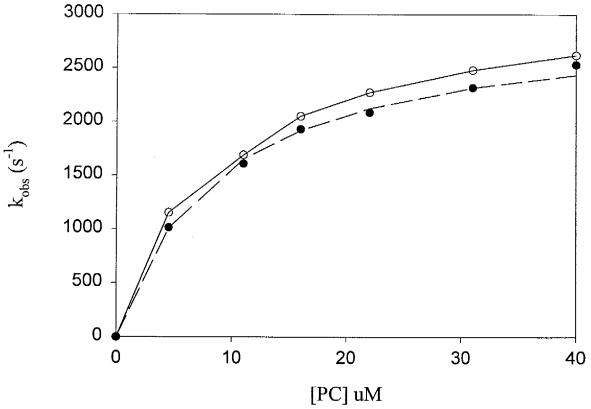


Fig. 2. Plot of k_{obs} versus plastocyanin concentration obtained with 5 μM Ru-13-cytochrome *c* and (●) = 5 mM phosphate and 0.5 mM EDTA and (○) = 2 mM phosphate and 10 mM aniline at pH 7. Solid line is plot of Eq. (1) with $K_a = 1.2 \times 10^5$ M^{-1} and $k_{et} = 3200$ s^{-1} and the dashed line was calculated using $K_a = 1.2 \times 10^5$ M^{-1} and $k_{et} = 2900$ s^{-1}.

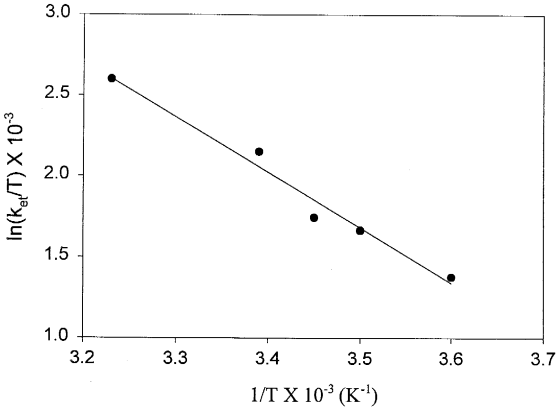


Fig. 3. Eyring plot of k_{et} over the temperature range of 3.5 to 37°C obtained with 5 μM Ru-13-cytochrome *c*, 5 mM phosphate and 10 mM aniline. [PC] was varied over the range of 10 to 40 μM to determine k_{et}.

ues of K_a and k_{et} obtained under a variety of solution conditions are summarized in Table 1. The data indicate that k_{et} shows a small, but statistically significant, increase with increasing ionic strength. Data collected with [phosphate] > 10 mM show insufficient curvature to allow a reliable fit to Eq. (2). Under these conditions the protein/protein association constant is very small and the reaction obeys simple pseudo-first-order kinetics in [PC] since [CC(II)] ≪ [PC(II)].

We have examined the dependence of the rates of electron transfer on temperature over the range of 3.5 to 37°C. The dependence of the observed rate constant on [PC] was also examined over this temperature range. At each temperature saturation kinetics was observed with [CC] = 5 μM and [PC] > 40 μM. The association constants and limiting rate constants were determined using Eq. (2). The temperature dependence of the limiting electron transfer rate constant is displayed in the form of an Eyring plot in Fig. 3. The activation parameters were independent of reaction conditions within the error limits of the determinations and are summarized in Table 1.

The viscosity dependence of the rate constant for electron transfer was determined by the addition of sucrose to the reaction solutions. Addition of up to 70% sucrose caused a four-fold decrease in k_{obs}. Limited measurements of the k_{obs} versus [PC] showed saturation behavior similar in form to that observed in the absence of sucrose. However, in solutions containing 64% sucrose, saturation occurred at slightly lower [PC] than in the absence of sucrose. The viscosity dependence of k_{obs} is shown in Fig. 4 and can be described by the simple inverse relation,

Table 1
Activation parameters [a], association constants [b] and rate constants [b] for electron transfer between Ru-13-CC and PC at pH = 7

Conditions	K_a (M^{-1})	k_{et} (s^{-1})	$\Delta H^{\pm}$ (cal mol^{-1})	ΔS^{*} (cal mol^{-1} K^{-1})
500 μM EDTA, no added buffer	$3\,(1) \times 10^5$	2600 (100)	6600 (1000)	−21 (2)
500 μM EDTA, 2 mM phoshate buffer	$1\,(0.5) \times 10^5$	3200 (100)	6500 (400)	−21 (2)
500 μM EDTA, 5 mM phoshate buffer	$1\,(0.7) \times 10^5$	3200 (100)	7500 (1000)	−18 (2)
10 mM Aniline, 2 mM phosphate buffer	$1\,(0.3) \times 10^5$	3000 (200)	6800 (1000)	−20 (2)
10 mM Aniline, 5 mM phosphate buffer	$7\,(2) \times 10^4$	3400 (300)	6800 (500)	−20 (2)

[a] Standard deviations are given in parentheses. [b] Association constants and rate constants were determined at 22°C.

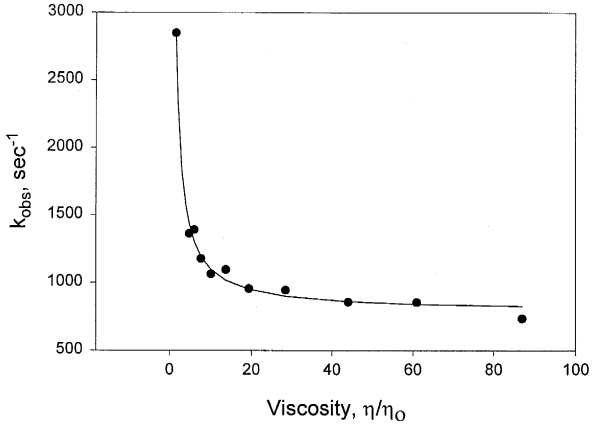


Fig. 4. Plot of k_{obs} for the photoinduced electron-transfer reaction between Ru-13-cyt *c* and plastocyanin as a function of viscosity. Solutions contained 10 μM Ru-13-cyt *c*, 15 μM PC, 0.5 mM EDTA, 2 mM phosphate buffer, pH 7, and sufficient sucrose to give the indicated viscosity. The solid line was calculated from the equation $k_{calc} = 3116/(\eta/\eta_0) + 791$.

$k_{obs} = A/(\eta/\eta_0) + B$, with a correlation coefficient > 0.99. Under the conditions of the experiments there was no indication that the sucrose was involved in the photochemical or thermal reactions

4. Discussion

In the context of the semiclassical description of electron-transfer reactions developed by Marcus [22], rate constant for electron transfer is related to the change in free energy of reaction according to Eq. (3).

$$k_{et} = \left(\frac{4\pi^3}{h^2\lambda k_B T}\right)^{1/2} (H_{AB})^2 \exp\left[-\frac{(\Delta G^{0\prime} + \lambda)^2}{4\lambda k_B T}\right] \quad (3)$$

The reorganization energy, λ, describes the energy required for structural changes in the reactants and reorientation and repolarization of the surrounding solvent prior to electron transfer. H_{AB} is a measure of the electronic coupling between the redox centers in the transition state. Marcus and Sutin [23] have shown that the reorganizational barrier for electron transfer is further related to the enthalpy of activation according to Eq. (4).

$$\Delta H^{\pm} = \frac{\lambda}{4} + \frac{\Delta H^{0\prime}}{2}(1 + \Delta G^{0\prime}/\lambda) + \frac{(\Delta G^{0\prime})^2}{4\lambda} \quad (4)$$

Since both $\Delta G^{0\prime}$ and $\Delta H^{0\prime}$ for the reaction of cytochrome *c* with PC are available from electrochemical measurements [18,19], we can calculate an apparent reorganization barrier for the reaction under investigation. The question of whether this measurement actually represents the reorganizational barrier to electron transfer rests on the assumption that the calculated first-order rate constant (k_{et}) describes a simple electron-transfer step. In the present case, application of Eq. (4) to the data in Table 1 (i.e., $\Delta H^{\pm} = 7$ kcal mol^{-1}) yields an apparent reorganizational barrier for electron transfer, $\lambda_{CC/PC}$, of 1.3 eV.

Marcus [22] has suggested that the reorganizational barrier of an electron-transfer reaction can be described in terms of intrinsic reorganizational barriers for each reactant through a simple cross-relation given by Eq. (5)

$$\lambda_{CC/PC} = \frac{\lambda_{CC} + \lambda_{PC}}{2} \quad (5)$$

where λ_{CC} and λ_{PC} are the intrinsic reorganizational barriers of CC and PC, respectively. Studies of cytochrome *c* indicate that $\lambda_{CC} = 1.0$ eV [24]. Using this value and $\lambda_{CC/PC} = 1.3$ eV obtained in the preceding paragraph, Eq. (5) yields $\lambda_{PC} = 1.6$ eV. This value seems to be unreasonably high in view of previous estimates of the intrinsic reorganizational barrier for PC. Other investigators [25,26], for example, have indicated that the intrinsic reorganizational energy for plastocyanin should be in the order of 1.0–1.2 eV. Several explanations of this difficulty are apparent: (1) the reorganizational barrier for plastocyanin is much higher than expected, (2) the cross-relation given by Eq. (5) is not appropriate, (3) the reorganizational energy for cytochrome *c* is significantly higher or (4) the rate of reaction is not limited by the electron-transfer step.

The apparent reorganizational barrier obtained above leads to further inconsistencies if it is used to calculate the electronic coupling in the CC/PC complex. Specifically, application of the free energy relation derived by Marcus shown in Eq. (3) yields an

electronic coupling term or tunneling matrix element, $H_{AB} = 0.30\ cm^{-1}$. The magnitude of this coupling is much larger than expected and is not in keeping with the distance between the redox centers in the CC/PC complex derived from modeling studies [8]. In modeling studies of the electrostatically stabilized complex, the heme-edge to Cys84 S distance is 16 Å and involves some through-space electronic coupling. For comparison, $H_{AB} = 0.26\ cm^{-1}$ for electron transfer between cytochrome b_5 and ruthenium polypyridine complexes covalently bound at Cys65 [27]. In this well-defined example, the edge-to-edge distance between the bipyridine ligands and His63 is only 12 Å and the redox centers are linked through 12 covalent bonds. The electronic coupling in the cytochrome b_5 example is in accord with a simple exponential distance model and more elaborate dominant pathways models.

The observed rate constants decline with increasing solution viscosity. If k_{obs} described a simple electron-transfer process in a protein complex, the reaction should be independent of viscosity. The observed dependence suggests the involvement of a diffusional process in the rate-limiting step in the overall electron-transfer reaction between CC and PC. Other explanations, such as a change in the dielectric constant of the solvent which may be responsible for the observed changes can be ruled out on the basis of work by Zhou and Kostić [13,14]. These investigators have addressed this question extensively in the context of the reaction between CC(Zn) and PC. In that case, the intracomplex quenching rate constants were shown to have the same viscosity dependence in ethylene glycol/water, glycerol/water or glucose/water solutions and were shown not to correlate with the dielectric constants of the solvents. The viscosity dependence reported in the present study and that presented by Zhou and Kostić [14] can both be described by the relation $k_{obs} = A/(\eta/\eta_0) + B$.

It appears that neither the $\Delta H^{\pm}$, the calculated electronic coupling H_{AB}, nor the viscosity dependence are consistent with those expected for a reaction limited by electron transfer in the electrostatically stabilized CC/PC complex. We conclude, therefore, that the reaction for which we have obtained rate constants is not rate-limited by electron transfer. Instead, we suggest that the reaction is controlled by reaction 9 in the following series of reactions.

$$\mathrm{PC(II)} + \text{Ru-Cyt } c\mathrm{(III)} \overset{k_{form}}{\rightarrow} \left[\mathrm{PC(II)},\text{Ru-Cyt } c\mathrm{(III)}\right]_s \quad (6)$$

$$\left[\mathrm{PC(II)},\text{Ru-Cyt } c\mathrm{(III)}\right]_s \overset{h\nu}{\rightarrow} \left[\mathrm{PC(II)},\text{Ru-Cyt } c\mathrm{(II)}\right]_s \quad (7)$$

$$\left[\mathrm{PC(II)},\text{Ru-Cyt } c\mathrm{(II)}\right]_s \overset{k_{ets}}{\rightarrow} \left[\mathrm{PC(I)},\text{Ru-Cyt } c\mathrm{(III)}\right]_s \quad (8)$$

$$\left[\mathrm{PC(II)},\text{Ru-Cyt } c\mathrm{(II)}\right]_s \overset{k_{sf}}{\rightarrow} \left[\mathrm{PC(II)},\text{Ru-Cyt } c\mathrm{(II)}\right]_f \quad (9)$$

$$\left[\mathrm{PC(II)},\text{Ru-Cyt } c\mathrm{(II)}\right]_f \overset{k_{etf}}{\rightarrow} \left[\mathrm{PC(I)},\text{Ru-Cyt } c\mathrm{(III)}\right]_f \quad (10)$$

$$\left[\mathrm{PC(I)},\text{Ru-Cyt } c\mathrm{(III)}\right]_f \overset{k_{dis}}{\rightarrow} \mathrm{PC(I)} + \text{Ru-Cyt } c\mathrm{(III)} \quad (11)$$

Reaction 9 describes the movement of cytochrome *c* from the electrostatically favored position within the CC/PC complex to a different location or orientation from which electron transfer is fast. This sequence is the same as that described by Zhou and Kostić [14] for reaction of zinc substituted cytochrome *c*. In this series of reactions the subscript 's' indicates the predominant form of the protein complex and the subscript 'f' indicates a form of the complex in which electron transfer is fast. Eqs. (6) and (7) describe the association of cytochrome *c* with PC and the subsequent photochemical reduction of the heme. The rate constant k_{ets} describes the rate of electron transfer in the predominant form of the electrostatically stabilized CC/PC complex. Work by Peerey and Kostić [11] with the covalently cross-linked CC/PC complex strongly suggests that k_{ets} is extremely small. The rate constant k_{etf} describes the rate of electron transfer in the fast reacting form of the protein/protein complex. In this reaction sequence we suggest that $k_{etf} \gg k_{sf} \gg k_{ets}$.

We offer the following arguments in support of the reaction sequence described by Eqs. (6)–(11). We note first that the comparison of the rate constant for

the reaction of cytochrome *c* labeled at Lys13 to that with native cytochrome *c*. Both modeling [8] and experimental studies [28] have shown that Lys13 is important in the formation of electrostatically stabilized complex with PC. The rate constant obtained with this derivative is nearly three times larger than the rate constant obtained with native horse cytochrome *c* (1000 s^{-1}; [1]). In terms of the above mechanism we suggest that the unfavorable placement of the bulky ruthenium label enhances formation of the fast reacting form of the complex relative to that of the native cytochrome *c*.

Similar comparisons can be made with the association constants. Specifically, the association constants for the native CC/PC complex are $> 5 \times 10^6$ M^{-1} at $\mu = 1$ mM and 8×10^4 M^{-1} at $\mu = 40$ mM [11,12]. These values, although not comparable on a one-to-one basis, are consistently larger than the association constants for the Lys13-labeled derivative shown in Table 1. The data indicate that Ru-13-CC forms a weaker protein/protein complex than native cytochrome *c* and implies that k_{dis} is larger in Ru-13-CC than in native CC.

Zhou and Kostić [14] in a very elegant study have convincingly demonstrated that electron transfer between PC and zinc or tin substituted cytochromes *c* are rate-limited by a process they describe as surface diffusion. The rate constant for this process is 2×10^5 s^{-1}. Simple dissociation of the protein/protein complex was ruled out on the grounds that it was at least an order of magnitude smaller and therefore could not be rate-limiting. Zhou and Kostić [14] extended these observations to the iron containing system and concluded that since the rate of surface diffusion was fast compared to the k_{ets} in the CC(Fe)/PC system then it would appear that the reaction in the native system is limited by electron transfer and that the measured rate constants are a good description of the electron-transfer process.

This conclusion is in conflict with the data presented in this report. We suggest the following rationalization of this difficulty. The problem is actually one of competitive processes and how these might change as a function of driving potential. We focus here on the diffusion step. In both systems, surface diffusion should be nearly identical and random. However, the rate of reaction will depend on how often surface diffusion brings the two redox centers to suitable separation distances. At any given separation distance the rate constant for electron transfer with the CC(Zn) will be larger than that with CC(Fe) because the former has a higher free energy of reaction. The difference will depend on the magnitude of λ but given $\lambda = 1$ eV, the rate constants for CC(Zn) will be $10^2 - 10^3$ larger than the rate constants for CC(Fe) at all separation distances. Accordingly, diffusional motion in the CC(Fe)/PC complex of the same magnitude as that sampled by the CC(Zn)/PC complex will not bring the Fe/Cu redox centers close enough to react at rates comparable to CC(Zn). The extent of surface diffusion in the CC(Fe)/PC complex must be larger and more time-consuming than in the zinc case. Therefore, the rate at which the reactants obtain suitable reaction distances (described by k_{fs}) must be smaller in the CC(Fe)/PC case. In keeping with this argument, Kostić and coworkers [11,12] have shown that electron transfer takes place in the covalently linked complex containing CC(Zn) but not CC(Fe).

If the diffusion distances are very large, then the rate of producing fluctuations large enough to sample productive electron transfer sites may be smaller than the rate of escape from the protein complex solvent cage. In this case the rate of electron transfer could be limited by dissociation from the protein-protein complex. At the present time we do not have sufficient evidence to suggest that k_{et} is equal to the rate constant for dissociation but we note that the measured rate constants are comparable to the expected rate constants of dissociation of the protein complex. For example, if we assume that formation of the protein-protein complex takes place with a diffusion limited rate constant of 1×10^9 M^{-1} s^{-1} and let $K_a = k_{diff}/k_{dis} = 2 \times 10^5$ M^{-1} (an average of the values given in Table 1) then $k_{dis} = 5000$ s^{-1}. Zhou and Kostić [14] suggested that $k_{dis} < 10000$.

In conclusion, the growing body of evidence strongly indicates that the electron-transfer reaction between native cytochrome *c* and plastocyanin is not limited by the electron-transfer step. The reaction exhibits a strong viscosity dependence and has activation parameters which are difficult to rationalize in terms of a simple electron-transfer reaction. The work by Peerey and Kostić [11] clearly shows that the covalent linked CC/PC complex is unreactive. Early ionic strength studies together with electric field cal-

culations by Rush et al. [29] indicated that reaction at the hydrophobic site around His87 were most consistent with the data.

Acknowledgements

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